

01 APR 2005

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
29 April 2004 (29.04.2004)

PCT

(10) International Publication Number  
WO 2004/035795 A1

(51) International Patent Classification<sup>7</sup>: C12N 15/63,  
A61K 39/12, 31/00

(21) International Application Number:  
PCT/KR2003/002163

(22) International Filing Date: 17 October 2003 (17.10.2003)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:  
10-2002-0063378 17 October 2002 (17.10.2002) KR

(71) Applicants (for all designated States except US): BI-  
OLEADERS CORPORATION [KR/KR]; 408-1,  
Sajeong-dong, Joong-gu, 301-212 Daejeon (KR). KO-  
REA RESEARCH INSTITUTE OF BIOSCIENCE  
AND BIOTECHNOLOGY [KR/KR]; 52, Eoeun-dong,  
Yuseong-gu, 305-806 Daejeon (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): SUNG, Moon-Hee

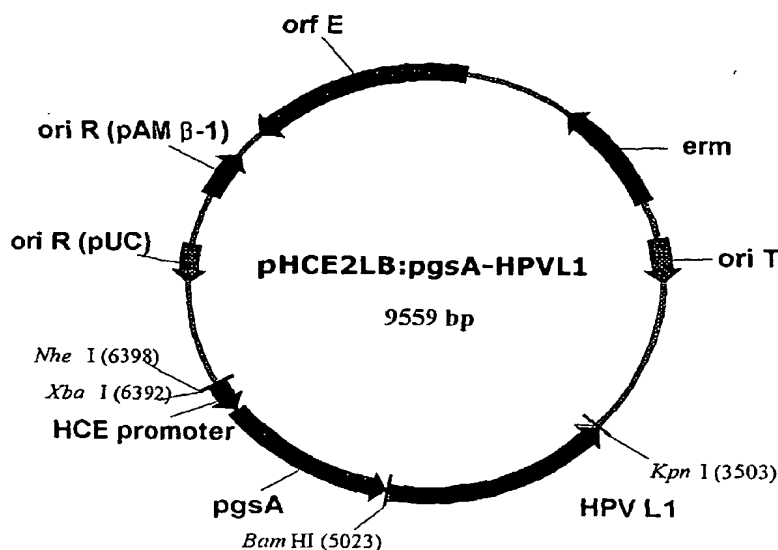
[KR/KR]; 908ho, Dong-A venture Bldg., 538-8, Bongmy-  
oung-dong, Yuseong-gu, 305-301 Daejeon (KR). POO,  
Ha-Ryoung [KR/KR]; 1619ho, Honors Vill, Dun-  
san-dong, Seo-gu, 302-121 Daejeon (KR). LEE, Jong-Soo  
[KR/KR]; 307-11, Jangdae-dong, Yuseong-gu, 305-308  
Daejeon (KR). JUNG, Chang-Min [KR/KR]; 618-13,  
Mok 3-dong, Yangcheon-gu, 158-811 Seoul (KR). HONG,  
Seung-Pyo [KR/KR]; 310-1503, Songgang Green APT.,  
Songgang-dong, Yuseong-gu, 305-751 Daejeon (KR).  
KIM, Chul-Joong [KR/KR]; 103-801, Hanwool APT.,  
Shinseong-dong, Yuseong-gu, 305-345 Daejeon (KR).  
PARK, Sue-nie [KR/KR]; 103-202, Inwangsan Hyundai  
APT., Hongje-dong, Seodaemoon-gu, 120-090 Seoul  
(KR). PYO, Hyun-mi [KR/KR]; 2-901 Garam APT.,  
Samcheon-dong, Seo-gu, 302-744 Daejeon (KR).

(74) Agent: KIM, Won-joon; 305, Soohyub Bldg., 917, Dun-  
san-dong, Seo-gu, Daejeon 302-828 (KR).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE,  
GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,

[Continued on next page]

(54) Title: VECTOR FOR ANTI-HPV VACCINE AND TRANSFORMED MICROORGANISM BY THE VECTOR



(57) Abstract: The current invention relates to expression vectors that can efficiently produce virion capsid protein, tumor-associated protein of human papillomavirus on a microbial surface and bacterial strains harboring such surface display vectors and the use of the bacterial strains or it's extract, purified products as complex vaccines. More particularly, the present invention relates to surface display vectors contains one or more than two genes selected among *pgsB*, *pgsC* and *pgsA*, encoding a poly- $\gamma$ -glutamic acid synthetase complex (*pgsBCA*) of a *Bacillus* sp. Strain and genes which encode virion capsid proteins, tumor-associated proteins of human papillomavirus, and method for preparing the same.

WO 2004/035795 A1



MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,  
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,  
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(84) **Designated States (regional):** ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,

**Published:**

— with international search report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**VECTOR FOR ANTI-HPV VACCINE AND TRANSFORMED  
MICROORGANISM BY THE VECTOR**

5

**TECHNICAL FIELD**

The present invention relates to a vector for expressing a vaccine  
against a surface antigen of papilloma virus, a microbial transformant and a  
10 vaccine using said microbial transformant or purified extract thereof.

**BACKGROUND**

15 Cell surface display is new technique by which a desired protein is  
expressed and attached onto a cell surface of microbe and is an application  
by using molecular biological information as the mechanism of protein  
secretion is elucidated. Precisely, the cell surface display uses a microbial  
cell surface protein derived from bacteria, yeast or the like as a surface  
20 anchoring motif to produce an exogenous protein on a cell surface. It has a  
broad range of application and thus, can be used to produce recombinant live  
vaccines, to prepare and screen a peptide/antigen library and to make whole  
cell absorbent, whole cell bioconversion catalyst and the like. Namely, this  
technique is considerably potential for an industrial use, since a variety of

exogenous protein expressed onto a cell surface decides the scope of industrial application.

Cell surface carrier is the most important factor to express an exogenous protein onto a cell surface successfully. To select an effective  
5 anchoring motif expressing an exogenous protein onto a cell surface is essential for this technique.

Accordingly, the surface expression carrier makes ready for several features as follows: Above all, some secretion signal is essential to help an exogenous protein pass through a cell inner membrane and finally arrive at a  
10 cell surface. Second, a targeting signal is required to anchor an exogenous protein stably onto a cell outer surface. Third, an anchoring motif seldom affects a cell growth even if massively expressed onto a cell surface. Fourth, an exogenous protein sustains the 3-dimensional structure to be expressed stably, regardless of a protein size. However, a surface expression carrier  
15 satisfying all the terms is not found yet and is complemented only to settle some disadvantage of above cases.

In a broad sense, the surface anchoring carrier is now classified to 4 kinds, including cell outer membrane protein, lipoprotein, secretory protein, surface organelle protein such as flagella protein. In case of Gram negative  
20 bacteria, a surface protein present on a cell outer membrane such as Lam B, Pho E (Charbit et al., J. Immunol., 139: 1658-1664, 1987; Agterberg et al., Vaccine, 8: 85-91, 1990), Omp A and the like is often exploited as a surface anchoring carrier. Besides, a lipoprotein such as Tra T (Felici et al., J. Mol. Biol., 222: 301-310, 1991), peptidoglycan associated lipoprotein (PAL)

(Fuchs et al., Bio/Technology, 9: 1369-1372, 1991), Lpp (Francisco et al., Proc. Natl. Acad. Sci. USA, 489: 2713-2717, 1992) and the like has been adopted and also fimbriae protein such as Fim A or Fim H adhesin of type I fimbriae (Hedegaard et al., Gene, 85: 115-124, 1989) and pili protein such as  
5 Pap A pilu subunit have been attempted to produce an exogenous protein. Furthermore, ice nucleation protein (Jung et al., Nat. Biotechnol, 16: 576-560, 1998; Jung et al., Enzyme Microb. Technol, 22(5): 348-354, 1998); Lee et al., Nat. Biotechnol., 18: 645-648, 2000), *Klebsiella oxytoca* pullulanase (Kornacker et al., Mol. Microl., 4: 1101-1109, 1990), *Neisseria* IgA protease  
10 (Klauser et al., EMBO L., 9: 1991-1999, 1990) or the like have been reported to become a surface anchoring motif. In case of Gram positive bacteria, it is known that a malaria antigen is effectively produced by using protein A derived form *Staphylococcus aureus* as a surface anchoring motif and that a surface coat protein from lactic acid bacteria is displayed well on a cell  
15 surface. Thus, the surface protein of Gram positive bacteria is verified to become a cell surface anchoring protein.

Previously, the present inventors have investigated a synthetic complex gene of poly- $\gamma$ -glutamate, pgs BCA derived from *Bacillus* sp. strain, as to whether it is applicable for new surface anchoring motif. Practically, pgs  
20 BCA gene was used to develop a novel recombinant vector expressing an exogenous protein onto a microbial surface and a method for producing an exogenous protein onto a cell surface in a large scale (Korean Patent Application No. 10-2001-48373).

Furthermore, the surface expression carriers described above have been tried a lot to produce various pathogenic antigens or antigenic determinants stably through the genetic engineering technique on a highly productive bacterial surface. Especially, it is reported that an exogenous immunogen can induce more consistent and stronger immune reactions when expressed onto a non-pathogenic bacterial surface and orally administered as a live vaccine, compared with typical vaccines such as detoxified pathogenic bacteria or virus.

Since the microbial surface structure acts as an adjuvant enhancing the antigenicity of exogenous protein expressed onto a cell surface, the immune reaction is known to be induced by live bacteria within a body. It is a remarkable event to develop a recombinant live vaccine of non-pathogenic bacterium through this surface expression system.

Human papilloma virus (hereinafter, referred to as "HPV") is presumed to world-widely infect more than 50% of all adult people. Especially, 4 types of HPV including HPV 16, 18, 31 and 45 are confirmed to cause a cervical cancer to more than 80% (Lowry D. R., Kirnbauer R., Schiller J. T., Proc. Natl. Acad. Sci., 91: 2436-2440, 1994). Papilloma virus is highly species-specific and small DNA tumor virus and belongs to family Papovaviridae which is infected to mammals such as human, cow, rabbit, sheep and so on and provokes a wart or papilloma on skin or mucosa (Pfister H., Adv. Cancer Res. 48: 113-147, 1987). Among these species, HPV are known to approximately 70 types and from more than 20 types cause tumors on skin mucosa of oral cavity or genital organs. Precisely, HPV 16 (type) and

HPV 18 are reported to cause cervical cancer mostly covering women cancers.

Cervical cancer is found frequently in women, next to breast cancer world-widely. WHO (World Health Organization) has reported that cervical cancer occurring newly is over 5 hundred thousand cases every year and more than 3 hundred thousand of patients are died from cervical cancer every year in the world. Especially in the developing country, cervical cancer is a major cause of women death (Pisani P., Parkin D. M., Ferlay J., Int. J. Cancer 55: 891-903, 1993). IARC statistics showed that the most effective way to eradicate papilloma virus infection is to administer a preventive vaccine for the future, since the developing country has even more chronic patients than the advanced country.

In order to develop certain vaccine against virus, the animal culture system should be equipped properly and exploited to produce and purify virus particles in a large scale. However, HPV is hardly changed to a virus particle *in vitro* or *in vivo*, since the virion is formed only in a fully differentiated keratinocyte. Thus, there are severe problems to develop vaccines against cervical cancer for the prevention and the treatment as well as to produce virus enough for this researches. In a broad sense, 2 types of vaccine including prophylactic vaccine and therapeutic vaccine are focused as a method for producing vaccines against cervical cancer. For a purpose, the prophylactic vaccine generates a stronger neutralizing antibody by HPV L1/L2 antigen and thus prevents a host from HPV infection. Even if already infected, it makes the disease no more progressed. In the meantime, the

therapeutic vaccine uses HPV E6/E7, induces specific cellular immune reactions and degenerates lesions or malignant tumor.

As searched out for last 20 years, HPV infected to human epithelium has various kinds of genotype and is associated with several benign and malignant tumors. Such an experimental data and discovery as to HPV promotes to develop HPV vaccine. HPV recombinant virus like particle is assumed more optimistic among several HPV vaccine candidates since better for an immune reaction than any other viruses except papilloma virus, through vaccine efficacy experiments in animal model and human (Koutsky L. A., Ault K. A., Wheeler C. M., Brown D. R., Barr E., Alvarez F. B., Chiacchierini L. M., Jansen K. U., N. Engl. J. of Med. 347(21): 1645-1651, 2002). Furthermore, HPV infection is confirmed recently to be the most essential cause of cancer definitely and scientists become interested in HPV studies and directly participate in this so as to accelerate the development of HPV vaccines globally. Nowadays, HPV vaccines widely known exploits HPV recombinant protein, HPV recombinant virus like particle, HPV DNA and the like to manufacture products.

In bacteria, yeast, animal cell and so on, the recombinant protein produced from some HPV partial composition through the recombinant DNA technology and a synthetic peptide in which some major epitope is synthesized chemically are tried to develop vaccines. Generally, recombinant proteins are produced through a common system such as bacteria, yeast, baculovirus, recombinant vaccinia virus and the like, by which several researches are accomplished to produce HPV recombinant proteins and to



identify the antibody forming ability against HPV in serum, the induction of cellular immune reaction and the like. However, the virus system using animal and insect cells is disadvantageous and thus contaminated during cultivation and hard to be purified. Including the synthetic peptide, overall cases cost high and are commercially limited in the industry, since papilloma virus infected patients are often found in the developing countries.

Practically, HPV L1 virus like particle (hereinafter, referred to as "VLP") has been disclosed to be produced as a live recombinant vaccinia virus through a mammary gland cell culture (Hagensee, M. E., Yaegashi, N., Gallowat, D. A., J. Virol. 67: 315-322, 1993) and VLP has been reported to generate neutralizing antibodies in a mouse model system (Schiller J. T., Lowry, D. R., Seminars in Cancer Biol. 7: 373-382, 1996). The therapeutic vaccine has been exploited by using HPV E6 and E7 protein uniquely expressed in cervical cancer (Bubenik J., Neoplasma 49: 285-289, 2002). In addition, HPV E6/E7 protein has been searched out as an immune target to treat cervical cancer and to develop a therapeutic vaccine, since it is a cancer specific antigen and associated with the cancerization of HPV infected cells. Actually, it is demonstrated that when HPV E6/E7 protein produced by the microbial system is administered to tumor cell – injected mice, the tumor formation is prevented and delayed (Gao L., Chain B., Sinclair C., J. Gen. Virol. 75: 157-164, 1994; Meneguzzi G., Cern C., Kieny M. P., Virology 181: 62-69, 1991). As shown in other cases, live virus vaccine has also problems to provoke an viral proliferation excessively and is liable to stay in a research level. Unfortunately, it takes a long time to be

commercialized and also needs considerable clinical trials. In order to overcome such a disadvantage, virus vectors which are inhibited or deficient in the replication are explored but not commercialized yet (Moss B., Proc. Natl. Acad. Sci. USA 93: 11341-11348, 1996).

5           On the other hand, vaccine studies by using bacterial vectors are proceeded actively. It is disclosed that HPV 16 VLP produced from attenuated *Salmonella typhimurium* inducibly generate antigen specific antibodies in mouse mucous membrane or whole body. Besides, vaccine composed of synthetic peptides uses only essential synthetic epitope enough  
10       to induce an immune reaction for the vaccination and particularly, an epitope inducing cytotoxic T lymphocyte (CTL) against HPV 16 E6/E7 has been already elucidated (Ressing M. E., Sette A., Brandt R. M., J. Immunol. 154: 5934-5943, 1995).

          In addition to such a trials, vegetables including tomatoes, potatoes  
15       and so on are utilized to produce viral antigens in plants and also a vegetable transformant itself is being attempted toward oral vaccine or edible vaccine. As a model case, hepatitis B surface antigen particle (Thavala Y. F. and C. J. Artzen. Proc. Natl. Acad. Sci. USA 92: 3358-3361) and capsid protein L1 and L2 of papilloma virus (Korean Patent Application 10-2000-0007022) are  
20       exemplified. In the plant system, however, HPV L 1 protein is expressed in so small amount and so hard for the purification to limitedly engaged in commerce.

          Therefore, since human papilloma virus is considered to be very often infected to people in the developing countries, in order to prevent and

effectively treat tumors derived from papilloma virus onto skin mucus of oral cavity or genital organs, it is deeply required to develop a novel method for preparing human papilloma viral antigens more economically and stably.

5

### **DISCLOSURE OF THE INVENTION**

In order to settle above-mentioned technical problems, the object of the present invention is to provide a recombinant vector and a microbial transformant which can produce HPV antigen through a microbial surface expression system.

In addition, another object of the present invention is to provide a vaccine for treating and preventing mucosal tumor which is comprised of a microbial transformant in which HPV antigen is expressed onto a cell surface, a crude HPV antigen extracted from the transformant or a HPV antigen purified from the microbial transformant as an effective component.

In order to attain the above-mentioned object, the present invention provides a surface expression vector for preparing a vaccine which contains one or more than two genes encoding poly- $\gamma$ -glutamate synthetase complex selected among pgs B, pgs C and pgs A and a surface antigen protein gene from human papilloma virus (HPV).

The gene pgs B, pgs C and pgs A mentioned in the present invention include nucleotide sequences comprising SEQ ID NO. 1, SEQ ID

NO. 2 and SEQ ID NO. 3 respectively.

The surface antigen protein gene of the present invention can be any kind of gene encoding a HPV surface component protein. For example, a capsid protein of human papilloma virus, HPV L1 or HPV L2 antigen  
5 protein gene can be used solely or more than two genes, coordinately. The major capsid, HPV L1 antigen protein gene is preferable to be used.

The tumor-associated antigen gene of the present invention can be any kind of gene encoding a tumor-associated protein of HPV and a tumor-associated antigen protein of human papilloma virus, HPV E6 or HPV E7  
10 antigen protein gene can be used solely or more than two genes can be used coordinately. Besides, the modified tumor-associated gene E6 and E7 can be used as an antigen and the major tumor-associated antigen protein of HPV, E7 antigen protein is preferable to be used.

More preferably, the gene encoding poly- $\gamma$ -glutamate synthetase  
15 complex of the present invention can be pgs A.

Furthermore, the present invention relates to a microbial transformant transformed with the recombinant vector for preparing a vaccine described above.

Any kind of microorganism can be applied for the present invention,  
20 if non-toxic to a living body or attenuated. For example, *Escherichia coli*, *Salmonella typhi*, *Salmonella typhimurium*, *Vibrio cholera*, *Mycobacterium bovis*, *Shigella* and the like can be selected as Gram negative bacterium and *Bacillus*, *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Listeria monocytogenesis*, *Streptococcus* and the like as Gram positive bacterium

properly.

The present invention provides various vaccines for treating and preventing mucosal tumors which exploits microbial transformant itself  
5 expressing an antigen protein onto a cell surface, or uses crude extract of cell membrane components after microbes are disrupted, or exploits an antigen protein purified from a microbes as an effective component. That is to say, the vaccine of the present invention can be used for a therapeutic drug or a prophylactic drug for treating or preventing a tumor which is  
10 caused by HPV and occurs on the mucosal membrane of oral cavity or genital organs, especially women cervical cancer.

The vaccine of the present invention can be administered orally or be edible, and injected hypodermically or peritoneally and can be a washing solution for genital organs. When administered directly to women genital  
15 organ, a host cell can be preferably selected from useful bacteria such as *Lactobacillus* sp. strain, which is clear to those skilled in this art.

Besides, the vaccine of the present invention can be sprayed for the nasal cavity.

The mucus immunization is very important to prevent HPV infection  
20 since the HPV infection is often caused onto the mucosal surface. The oral vaccine using a microbial transformant itself is expected to be more effective to prevent HPV than the parental vaccine, since a microbe expressing HPV antigen onto a cell surface is advantageous to induce a mucosal response on the membrane more efficiently.

Concretely, the present invention provides the recombinant vector for a vaccine use, pHCE2LB:pgsA-HPV L1 (See Fig. 1) which contains pgs A gene, a poly- $\gamma$ -glutamate synthetase complex gene derived from *Bacillus* sp. strain and expresses HPV L1 protein, a fused protein connecting the C-terminus of pgs A and the N-terminus of HPV L1 onto a cell surface of Gram negative bacterium or Gram positive bacterium and also the microbial transformant for the surface expression. The *Escherichia coli* transformant transformed with the above-mentioned vector for a vaccine use has been deposited separately (accession number: KCTC 10349 BP).

In addition, the present invention provides the surface expression recombinant vector for a vaccine use, pHCE2LB:pgsBCA-HPV E7 (See Fig. 5) which contains pgs BCA gene, a poly- $\gamma$ -glutamate synthetase complex gene derived from *Bacillus* sp. strain and expresses HPV E7 protein, a fused protein connecting the C-terminus of pgs A and the N-terminus of HPV E7 onto a cell surface of Gram negative bacterium or Gram positive bacterium and also the microbial transformant for the surface expression. The *Escherichia coli* transformant transformed with the above-mentioned vector for a vaccine use has been deposited separately (accession number: KCTC 10520 BP).

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

The above and other objects, features and other advantages of the

present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which;

**FIG. 1** depicts the genetic map of the recombinant vector  
5 pHCE2LB:pgsA-HPV L1 which uses Gram negative bacterium and Gram  
positive bacterium as a host cell for the surface expression.

**FIG. 2A and 2B** depicts the expression of HPV L1 antigen fused with  
pgs A within *Salmonella* strain and *Lactobacillus* strain transformed with the  
10 recombinant vector pHCE2LB:pgsA-HPV L1 for the surface expression.

**FIG. 3A** depicts the IgG antibody titer against HPV L1 antigen in  
serum from mouse in which *Lactobacillus casei* transformed with the  
recombinant vector pHCE2LB:pgsA-HPV L1 of the present invention and  
15 then verified to express the antigenic determinant onto a cell surface, is orally  
administered in a proper amount for some time.

**FIG. 3B** depicts the IgG antibody titer against HPV L1 antigen in  
mouse washing solution of intestine, bronchial tube, lung and vagina to which  
20 *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsA-  
HPV L1 and then verified to express the antigenic determinant onto a cell  
surface, is orally administered in a proper amount for some time.

**FIG. 4** depicts the cytotoxic activity of cytotoxic T lymphocyte in a

spleen cell obtained from mouse in which *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsA-HPV L1 and then verified to express the antigenic determinant onto a cell surface, is orally administered in a proper amount for some time.

5

**FIG. 5** depicts the genetic map of the recombinant vector pHCE2LB:pgsBCA-HPV E7 for the surface expression which uses Gram negative bacterium and Gram positive bacterium as a host cell.

10

**FIG. 6** depicts the expression of HPV E7 antigen fused with pgs A in *Lactobacillus* strain which is transformed with the recombinant vector pHCE2LB:pgsBCA-HPV E7 for the surface expression, by performing western blotting with specific antibodies.

15

**FIG. 7** depicts the proliferation rate of tumor cells obtained from mouse in which *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsBCA-HPV E7 and then verified to express the antigenic determinant onto a cell surface, is orally administered in a proper amount for some time and challenged according to a time lapse.

20

**FIG. 8** depicts the genetic maps of the cloning vector pGNBCA and the recombinant vector pGNBCA-HB168 for the surface expression as described in Indirect Example of the present invention.



**FIG. 9** depicts the surface expression of hepatitis B virus surface antigen protein from Gram negative bacterium transformed with the recombinant vector pGNBCA-HB168 by performing western blotting and fluorescent activating cell sorting flow cytometry as described in Indirect Example of the present invention.

**FIG. 10** depicts the genetic maps of the cloning vector pGNCA and the recombinant vector pGNCA-HB168 for the surface expression as described in Indirect Example of the present invention.

**FIG. 11** depicts the surface expression of hepatitis B virus surface antigen protein from Gram negative bacterium transformed with the recombinant vectors (pGNCA-HB168:A2, pGNA-HB168:A3 and pGNHB-A:A4) by performing western blotting and fluorescent activating cell sorting flow cytometry as described in Indirect Example of the present invention.

## **EXAMPLES**

Practical and presently preferred embodiments of the present invention are illustrated as shown in the following Examples.

However, it will be appreciated that those skilled in the art, on consideration of this disclosure, may make modifications and improvements within the spirit and scope of the present invention.

Especially, HPV type 16 L1 antigen protein gene is adopted in following Examples, but any kind of antigen protein gene, a HPV capsid protein gene such as L1, L2 or so on from any other types of HPV strains can be used solely or more than two coordinately. In following Examples, HPV type 16 E7 gene, a major antigen protein gene associated with a cancer induction is used but any other cancer inducing antigen protein gene of other HPV strains can be selected solely or coordinately.

Besides in following Examples, the cell outer membrane protein gene, pgs BCA participating in the poly- $\gamma$ -glutamate synthesis is collected from *Bacillus subtilis* var. chungkookjang (accession number: KCTC 0697 BP), but the vector including all the kinds of pgs BCA gene from *Bacillus* sp. strain producing poly- $\gamma$ -glutamate, microbial transformants using the vector or the like can be within the scope of the present invention. Precisely, other pgs BCA genes having more than 80% homology of sequences present in the pgs BCA of *Bacillus subtilis* chungkookjang and derived from other microbial strains can be used to construct the vector for a vaccine use, which may be within the scope of the present invention.

As demonstrated in following Examples and Indirect Examples, pgs BCA gene can be utilized all or partially to construct the vector for a vaccine use, which may be within the scope of the present invention.

Furthermore in following Examples, *Salmonella typhi* as Gram negative bacterium and *Lactobacillus* as Gram positive bacterium are used as a host cell for the above-mentioned vector, but in addition to these bacteria, other Gram positive or Gram negative bacteria can be used for the

same result, which is clear to those skilled in this art.

Also in following Examples, the microbial transformant itself transformed with the vector for a vaccine use is only disclosed to apply for a living body as a live vaccine. However, according to general information in vaccine associated technical fields, it is natural to obtain the same or similar results even if crude extract from the above microbial transformant or purified proteins are administered into a living body.

**<Example 1> Construction of the recombinant vector pHCE2LB : pgsA : HPV L1 for the surface expression**

Cell outer membrane protein derived from *Bacillus* sp. strain and participating in the synthesis of poly- $\gamma$ -glutamate is exploited. Among the cell outer membrane gene, pgsBCA, gene pgsA was used to prepare the recombinant vector pHCE2LB:pgsA-HPV L1 which can express the major capsid protein L1 of human papilloma virus Type 16 (hereinafter, referred to as "HPV") onto a cell surface by using Gram negative bacteria and Gram positive bacteria as a host cell.

Above all, the gene encoding HPV L1 is introduced into the surface expression vector pGNA which uses Gram negative bacteria as a host cell (obtained from an applicant of Korean Patent Application No. 10-2001-48373). Precisely, approximately 1.5 kb of human papilloma virus gene cloned in pUC19 was utilized as a template and oligonucleotides encoding HPV L1 and containing the nucleotide sequence of SEQ ID. NO. 4 or SEQ ID.

NO. 5 as a template and then the polymerase chain reaction was performed.  
As a result, 1518 bp - sized gene was amplified.

The primers of SEQ ID. NO. 4 and SEQ ID. NO. 5 were made to include the recognition site of restriction enzyme Bam HI and Hind III present in the cloning vector pGNA for the surface expression. The HPA L1 antigen gene amplified above was digested with the restriction enzyme Bam HI and Hind III and ligated and adjusted in translation codons to the C-terminal region of cell outer membrane protein gene pgsA which participates in the synthesis of poly- $\alpha$ -glutamate and is derived from the cloning vector pGNA so as to manufacture the recombinant vector pGNA-HPV L1.

In order to obtain the DNA fragment containing HCE promoter, pgsA and HPV L1 from the recombinant vector prepared above, the recombinant vector pGNA-HPV L1 was digested with the restriction enzyme Nhe I and Sca I and the resulting fragment was inserted to the restriction enzyme Xba I and Sma I site within a multi-cloning site of common cloning vector pAT19 for Gram positive bacteria so as to construct the recombinant vector pHCE2LB:pgsA-HPV L1 (See Fig. 1).

The recombinant vector for the surface expression in the present invention was transformed to *Escherichia coli* and the bacterial transformant including pHCE2LB:pgsA-HPV L1 has been deposited to Korea Research Institute of Bioscience and Biotechnology, Gene Bank (KCTC, Taejon-si, Eusung-gu, Eoun-dong 52) with the accession number KCTC 10349 BP.

#### <Example 2> Surface Expression of HPV L1 fused with pgsA

Gram negative bacterium *Salmonella typhi* Ty21a was transformed with the recombinant vector pHCE21LB:pgsA-HPV L1 for the surface expression described above and examined the protein expression of HPV L1 antigen fused with pgsA in *Salmonella typhi* Ty21a. Then, Gram positive bacterium *Lactobacillus* was transformed, identified the presence of the recombinant vector pHCE21LB:pgsA-HPV L1 within *Lactobacillus* strain and examined the protein expression of HPV L1 antigen fused with pgsA (See Fig. 2). The bacterial expression of HPV L1 antigen associated with the C-terminus of pgsA gene participating in the synthesis of poly- $\gamma$ -glutamate was examined by performing SDS-polyacrylamide gel electrophoresis and western blotting with specific antibodies against HPV L1.

Concretely, *Salmonella typhi* Ty21a transformed with the recombinant vector pHCE2LB:pgsA-HPV L1 was cultured onto 500 mL flask including 50 mL of LB medium (yeast extract 5 g/L, tryptone 10 g/L, salt 5 g/L, pH 7.0) containing 100 mg/L of antibiotic (erythromycin) and induced for the surface expression. In addition, *Lactobacillus casei* was transformed with the recombinant vector pHCE2LB:pgsA-HPV L1, cultured onto MRS medium (*Lactobacillus* MRS, Becton Dickinson and Company Sparks, USA) at 37°C in static culture, proliferated and induced for the surface expression.

Afterward, *Salmonella typhi* Ty21a and *Lactobacillus casei* were induced for the surface expression and proteins were obtained in the same cell density, and denatured for sample uses. Then the proteins were analyzed through SDS-polyacrylamide gel electrophoresis and in a fractionated state, transferred onto PVDF membrane (polyvinylidene-

5 difluoride membrane: Bio-Rad). The resulting PVDF membrane was submerged in a blocking buffer (50 mM Tris-HCL, 5% skim milk, pH 8.0), shaken for 1 hour to be blocked, and reacted for 12 hours with a monoclonal primary antibody against HPV L1 proteins which was derived from mouse and diluted to 1,000 fold in the blocking buffer. The completely reacted membrane was washed with the buffer and reacted for 4 hours with a mouse secondary antibody which was conjugated with biotins and diluted to 1,000 fold in the blocking buffer. Then, the resulting membrane was washed with the buffer, reacted with avidin-biotin reagent for 1 hour and washed again.

10 The washed membrane became chromophored by adding H<sub>2</sub>O<sub>2</sub> and DAB solution and was identified to have a specific binding between specific antibody against HPV L1 and the above fused protein.(See Fig. 2)

In Fig. 2A, lane 1 is *Salmonella typhi* Ty21a, host cell not transformed and lane 2, 3 is *Salmonella typhi* Ty21a transformed with the recombinant vector pHCE2LB:pgsA-HPV L1. In Fig. 2B, lane 1 is *Lactobacillus casei* not transformed and lane 2 is *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsA-HPV L1. As illustrated in Figs, the fused protein expressed from the recombinant vector pHCE21LB:pgsA-HPV L1 was verified at 97.4 Kda band. Since pgsA has about 41.8 KDa and L1 protein, about 55.6 Kda, the band having above 97.4 Kda is elucidated as a fused protein of pgsA and L1 protein.

15

20




<Example 3> Induction of immune reaction in *Lactobacillus* expressing HPV L1 antigen onto cell surface

The recombinant vector pHCE2LB:pgsA-HPV L1 for the surface expression was transformed to Gram positive bacterium, *Lactobacillus casei*, induced to express the antigen onto cell surface of *Lactobacillus casei* as described in Example 2 and then the cell outer membrane protein pgsA participating in the synthesis of poly- $\gamma$ -glutamate and the HPV16 fused with L1 antigen were examined for the antigenicity.

Concretely, the recombinant vector pHCE2LB:pgsA-HPV L1 of the present invention was transformed to *Lactobacillus casei* for the surface expression, harvested to adjust each cell to the same concentration and washed several times with PBS buffer (pH 7.4). Then, *Lactobacillus* in which HPV16 L1 antigen is expressed onto cell surface and *Lactobacillus* not transformed were administered in  $5 \times 10^{10}$  bacteria to an oral cavity of 4~6 week BALB/c mouse, three times every other day in the first week and the second week and injected additionally after 2 weeks, three times every other day. As a standard group, mice which were administered with HPV 16 L1 virus like particle (VLP) expressed from yeast twice every other day were used.

In a 2-week interval after orally administered and venous injected, mice were victimized. Then, sera of each mouse group were collected to measure IgG antibody titers against HPV 16 L1 antigen within serum and suspension solutions washing internal organs, bronchial tube, lung and vagina were tried to estimate IgA antibody titers against HPV 16 L1 antigen by using ELISA method (Enzyme-linked immunosorbent assay) (See Fig 3a, 3b). For the ELISA method calculating IgG and IgA antibody titers against

HPV 16 L1 antigen, HPV 16 L1 virus like particle (VLP) expressed from yeast was used as an antigen. Precisely, for measuring IgG antibody titer, horseradish peroxidase conjugated anti-mouse IgG and for calculating IgG antibody titer, horseradish peroxidase conjugated anti-mouse IgA were utilized.

In Fig. 3a, ( ) is a group not transformed and orally administered with *Lactobacillus casei*; ( ) a group intravenously injected with L1 virus like particle of HPV 16 (VLP); and ( ) a group orally administered with *Lactobacillus casei* transformed with the vector pHCE2LB:pgsA:HPV. In Fig. 3b,  is a group not transformed and orally administered with *Lactobacillus casei*;  a group intravenously injected with L1 virus like particle of HPV 16 (VLP); and  a group orally administered with *Lactobacillus casei* transformed with the vector pHCE2LB:pgsA:HPV.

Consequently as illustrated in Fig. 3a, the serum solution which was obtained from BALB/c mouse group administered with *Lactobacillus casei* transformed with the vector pHCE2LB:pgsA-HPV L1 and diluted was confirmed to have a considerably higher IgG antibody titer against human HPV L1 antigen than that of a standard BALB/c mouse group, and to increase the antibody titers, which can be compared with those of a group intravenously administered with HPV 16 L1 virus like particle (VLP).

As illustrated in Fig. 3b, the suspension solution which washed internal organs, bronchial tube, lung, the internal part of vagina or the like of BALB/c mouse group administered with *Lactobacillus casei* transformed with the vector pHCE2LB:pgsA-HPV L1 and diluted, was confirmed to increase



IgG antibody titer against human HPV L1 antigen, which can be compared with those of the standard group intravenously administered with HPV 16 L1 virus like particle (VLP). Especially, the suspension of internal organ had the more remarkable IgA antibody titer against HPV 16 L1 antigen.

5           Accordingly, the microbial transformant of the present invention was verified to produce IgG antibody against HPV as an index of whole body immune induction and also IgA antibody against HPV as an index of limited immune, mucose immune induction and thus, the microbial transformant can be applied for a live vaccine.

10

**<Example 4> Cytolysis activity of spleen cell from experimental animal immunized with *Lactobacillus* expressing HPV L1 antigen onto cell surface**

15           In order to investigate whether a cell-mediated immune reaction is also induced among immune reactions through the same process, splenocytes were separated from the experimental mice immunized with *Lactobacillus* expressing HPV L1 antigen onto a cell surface and measured as to the activity of cytotoxic lymphocyte (CTL).

20           Concretely, the splenocyte of Balb/c mouse not immunized and 10 µg of synthetic HPV 16 L1 peptide were incubated together at 37°C for 3 hours and irradiated with 4,000 rad so as to prepare stimulating cells.

          As described in Example 3, the splenocytes of Balb/c mice immunized with *Lactobacillus* expressing HPV L1 antigen onto a cell surface were separated, mixed with a stimulating cell in which a synthetic peptide

was loaded and cultivated for 6 days so as to prepare an effector cell. At this moment, the cell ratio of stimulating cell and effector cell was adjusted to 2 : 1 and additionally stimulated in 6 days. Before one day for an assay, cells used for a target cell were incubated by adding 10 µg of synthetic HPV 16 L1 peptide. For the day of assay,  $^{51}\text{CrO}_4$  was added to peptide-loaded target cell in 100 µCi/ $10^6$  cell, incubated for 2 hours and washed. The effector cell prepared was allotted to a 96 well plate in a ratio to 5,000 cells/well target cell for the range of 1 : 100 ~ 1 : 25. The target cell and the effector cell were mixed and incubated at 37°C for 4 hours. After 4 hours, supernatant was collected to measure the release of  $^{51}\text{CrO}_4$ . The maximum release was at the point that 1% Triton X-100 and target cells were blended and the spontaneous release at the point that media and target cells were mixed and then the specific lysis was converted according to a following formula.

$$[\text{Specific lysis} = 100 * (\text{experimental cpm} - \text{spontaneous cpm}) / \text{maximum cpm} - \text{spontaneous cpm}]$$

The converted result of cytolysis activity was illustrated in Fig. 4.

Consequently as depicted in Fig. 4, the splenocyte of Balb/c mouse which was administered with *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsA-HPV L1 was identified to have a higher ratio of specific cytolysis than that of Balb/c mouse which was administered with *Lactobacillus* not transformed.

Therefore, the immune reaction induced by *Lactobacillus* expressing

HPV 16 L1 antigen of the present invention was verified to be a cell-mediated immune reaction which activates a cytotoxic lymphocyte as a major effect of an oral vaccine.

5     **<Example 5> Construction of the recombinant vector pHCE2  
LB:pgsBCA:HPV E7 for the surface expression**

10     The cell outer membrane protein derived from *Bacillus sp.* strain and participating in the synthesis of poly- $\gamma$ -glutamate is exploited. Among the cell outer membrane gene, pgs BCA gene was used to prepare the recombinant vector pHCE2LB:pgsA-HPV-E7 which can express the major antigen protein associated with a cancer induction E7 of HPV 16 onto a cell surface by using Gram negative bacteria and Gram positive bacteria as a host cell.

15     Above all, the gene encoding HPV 16 E7 is introduced into the surface expression vector pGNBCA which uses Gram negative bacteria as a host cell (obtained from an applicant of Korean Patent Application No. 10-2001-48373). Precisely, approximately 324 bp of human papilloma virus gene cloned in pUC19 was utilized as a template and oligonucleotides encoding HPV 16 E7 and containing the nucleotide sequence of SEQ ID. NO. 6 or  
20     SEQ ID. NO. 7 as a template and then the polymerase chain reaction was performed. As a result, 324 bp - sized gene was amplified.

The primers of SEQ ID. NO. 6 and SEQ ID. NO. 7 were made to include the recognition sites of restriction enzyme Bam HI and Hind III present in the cloning vector pGNBCA for the surface expression. The HPV

L1 antigen gene amplified above was digested with the restriction enzyme Bam HI and Hind III and ligated and adjusted in translation codons to the C-terminal region of cell outer membrane protein gene pgsA which participates in the synthesis of poly- $\gamma$ -glutamate and is derived from the cloning vector pGNBCA so as to manufacture the recombinant vector pGNBCA-HPV E7.

In order to obtain the DNA fragment containing HCE promoter, pgsBCA and HPV L1 from the recombinant vector pGNBCA-HPV E7 prepared above, the recombinant vector was digested with the restriction enzyme Nhe I and Sca I and the resulting fragment was inserted to the restriction enzyme Xba I and Sma I site within the multi-cloning site of common cloning vector pAT19 for Gram positive bacteria so as to construct the recombinant vector pHCE2LB:pgsBCA-HPV E7 (See Fig. 5).

The recombinant vector for the surface expression in the present invention was transformed to *Escherichia coli* and the bacterial transformant including pHCE2LB:pgsBCA-HPV L1 has been deposited to Korea Research Institute of Bioscience and Biotechnology Gene Bank (KCTC, Taejon-si, Eusung-gu, Eoun-dong 52) with the accession number KCTC 10520 BP.

#### **<Example 6> Surface Expression of HPV E7 fused with pgsA**

Gram negative bacterium *Lactobacillus* was transformed with the recombinant vector pHCE21LB:pgsBCA-HPV E7 for the surface expression described above, identified the presence of the recombinant vector pHCE21LB:pgsBCA-HPV E7 within *Lactobacillus* strain and examined the

protein expression of HPV E7 antigen fused with pgsA (See Fig. 6).

For this purpose, the surface expression vector was transformed to *Lactobacillus* and induced through the same process described in Example 2. Then, the expression of HPV E7 antigen protein fused with the C-terminus of pgsA gene participating in the synthesis of poly- $\gamma$ -glutamate was confirmed by performing SDS-polyacrylamide gel electrophoresis and western blotting with specific antibodies against pgsA (See Fig 6A) and HPV E7 (See Fig 6B) (See Fig. 6). In Fig. 6, lane 1 is *Lactobacillus casei* not transformed; and lane 2 and 3 are *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsBCA-HPV E7.

As illustrated in Figs., about 60.8 Kda band of fused protein was verified from the recombinant vector pHCE2LB:pgsBCA-HPV E7. Since pgs A has about 41.8 Kda and HPV 16 E7 antigen protein has about 19 Kda typically, the above 60.8 Kda band was elucidated to a fused protein of pgs A and HPV 16 E7 antigen protein.

**<Example 7> Challenge of tumor cell to experimental animal immunized with *Lactobacillus* expressing HPV E7 antigen onto a cell surface**

The recombinant vector pHCE21LB:pgsBCA-HPV E7 for the surface expression was transformed to Gram positive bacterium, *Lactobacillus casei*, induced to express the above antigen onto the surface of *Lactobacillus casei* through the same process described in Example 2 and then the inhibition effect upon the tumor proliferation was examined by the immunization of HPV

16 E7 antigen fused with cell outer membrane protein, pgs A participating in the poly- $\gamma$ -glutamate synthesis.

Concretely, *Lactobacillus casei* transformed with the recombinant vector pHCE2LB:pgsBCA-HPV E7 for the surface expression and  
5 *Lactobacillus* not transformed were treated as described in Example 3 and  $5 \times 10^{10}$  transformants were orally administered to 4 ~ 6 week - C57/BL/6 mouse three times in the first week and the second week every other day and after 2 weeks, additionally injected three times every other day.

After last injected, tumor cell line TC-1 ( $1 \times 10^4$  / 50  $\mu$ l) expressing  
10 HPV E7 was injected subcutaneously on the left side of mouse to be challenged.

Tumor cell line TC-1 expressing HPV E7 protein is induced from the primary lung cell of C57/BL/6 mouse which is immunized and transformed with HPV 16 E6 and E7 gene and activated human c-Ha-ras gene as  
15 demonstrated in the prior art [Lin et al., Cancer Res. 56:21-26, 1996]

In 2 weeks after tumor challenged, the tumor size was measured. Three times a week every other day, the longest and the shortest length were calculated and indicated to be doubled. The data measuring the tumor size after tumor challenged were illustrated in Fig. 7.

20 As shown in Fig. 7, tumor cell challenged with C57/BL/6 mouse which was administered with *Lactobacillus casei* transformed with the vector pHCE2LB:pgsBCA-E7 was remarkably inhibited to proliferate, compared with than the result of C57/BL/6 mouse administered with *Lactobacillus* not transformed.

Therefore, it is confirmed that the immune reaction induced by *Lactobacillus* in which HPV16 E7 antigen of the present invention was expressed onto a cell surface prevent tumor cells from the proliferation.

5     **<Indirect Example> Construction of the recombinant vector for a vaccine combining pgs B, pgs C and pgs A and experiment for expressing exogenous protein onto a cell surface by using the system**

It is confirmed that the surface expression vector can be constructed  
10    to include any one or more than two genes among pgs B, pgs C and pgs A encoding poly- $\gamma$ -glutamate synthetase complex and gene encoding exogenous protein and transformed to microbes so as to be expressed onto the microbial surface.

Accordingly, it is also identified indirectly that the vector for a vaccine  
15    use including any one or more than two genes among pgs B, pgs C and pgs A encoding poly- $\gamma$ -glutamate synthetase complex and gene encoding HPV antigen protein of the present invention can be manufactured.

In Indirect Example, the plasmid pGNBCA and pGNCA is the same  
20    with the plasmid vector pGNpgsBCA and pGNpgsCA of the present invention respectively.

**<Indirect Example 1> Construction of the recombinant vector pGNBCA-HB168 and surface expression of antigenic determinant forming neutralizing antibody of S antigen**

Cell outer membrane gene, pgs BCA participating in poly- $\gamma$ -glutamate synthesis derived from *Bacillus* sp. strain was utilized to construct the recombinant vector pGNBCA-HB168 expressing antigenic determinant forming neutralizing antibody of hepatitis B virus S antigen onto the cell surface by using Gram negative bacterium as a host cell.

In order to introduce hepatitis B virus S antigen gene to the surface expression vector pGNBCA by using Gram negative bacterium as a host cell, approximately 1.4 kb of hepatitis B virus gene cloned in the common cloning vector pUC8 was used as a template and oligonucleotides containing the nucleotide sequences of SEQ ID NO. 8 and SEQ ID NO. 9 as a primer. Then S antigen gene was amplified by performing the polymerase chain reaction. At this moment, the amplified gene is 168 bp in the size.

The primers of SEQ ID NO. 8 and SEQ ID NO. 9 described above were made to include the recognition site of restriction enzyme Bam HI and Hind III present in the surface expression vector pGNBCA. The hepatitis B virus S antigen gene amplified above was digested with the restriction enzyme Bam HI and Hind III and ligated to the C-terminus of cell outer membrane gene participating in poly- $\gamma$ -glutamate synthesis, previously prepared and adjusting the translation codon. As a result, the recombinant vector pGNBCA-HB168 was prepared as described above (See Fig. 8)

The recombinant vector pGNBCA-HB168 for the surface expression was utilized to express the antigenic determinant forming neutralizing antibody of hepatitis B virus S antigen in *Escherichia coli* and to examine the surface expression.



The surface expression vector constructed in Example 2 was transformed to *Escherichia coli* and cultivated in 50 mL of LB medium (yeast extract 5 g/L, tryptone 10g/L, salt 5g/L, pH 7.0) containing 100 mg/L antibiotic (ampicillin) and then induced for the surface expression.

5        The antigenic determinant forming neutralizing antibody of S antigen fused with the C-terminus of poly- $\chi$ -glutamate synthetase gene was expressed in bacteria and identified by performing SDS-polyacrylamide gel electrophoresis and western blotting with specific antibodies against S antigen. Concretely, proteins obtained in the same cell concentration were  
10        denatured to prepare a sample and analyzed by performing SDS-polyacrylamide gel electrophoresis and then, fractionated proteins were transferred to PVDF membrane. The PVDF membrane completed to transfer proteins was shaken and blocked for 1 hour by using blocking buffer (50 mM Tris HCl, 5% skin milk, pH 8.0). Afterward, the polyclonal primary antibody  
15        against S antigen derived from sheep was diluted to 1,000 X in blocking buffer and reacted for 12 hours. The reacted membrane was washed with buffer solution and incubated for 4 hours by diluting the secondary antibody against sheep conjugated with biotin in blocking buffer to 1,000 X. The  
20        reacted membrane was washed with buffer solution and incubated with avidin-biotin reagent for 1 hour and again washed. Onto the washed membrane, H<sub>2</sub>O<sub>2</sub> and DAB solution were added as a substrate and coloring reagent and became colored so as to identify the specific binding in between specific antibody against S antigen and the above mentioned fused protein (See Fig. A). In Fig. 9, lane 1 is host cell JM109 not transformed and lane 2 is

the transformant pGNBCA-HB168 / JM 109. As illustrated in Figs, approximately 48 KDa band of fused protein was verified by the plasmid pGNBCA-HB168.

In order to confirm the direct expression of the antigenic determinant forming neutralizing antibody of S antigen, *Escherichia coli* induced was separated respectively to soluble, inner membrane, outer membrane through the outer membrane fractionation method and then, identified by performing SDS-polyacrylamide gel electrophoresis and western blotting with antibodies against S antigen. Concretely, *Escherichia coli* transformant induced to express the fused protein onto a cell surface and *E. coli* not transformed were harvested with adjusting to the same cell concentration, washed in buffer solution (10 mM HEPES buffer, pH 7.4) several times, suspended in buffer solution containing 10 µg/ml lysozyme, 1 mM PMSF and 1 mM EDTA and reacted for 4 ~ 10 minutes. Afterward, DNase (0.5 mg/µl) and RNase (0.5 mg/ml) were added and cells were sonicated, separated to *E. coli* and cellular debris by centrifuging at 10,000 x g for 4, 20 minutes and collected the fraction including periplasm and cytoplasm of *E. coli*. Then, the collected pellet was suspended in PBS buffer (pH 7.4) containing 1% Sarcosyl (N-lauryl sarcosinate, sodium salt), separated to inner membrane protein of *E. coli* in a supernatant and to outer membrane protein in a cell pellet by centrifuging at 15,000 x g for 4, 2 hours and thus the fractions were obtained. Each fraction was identified by performing polyacrylamide gel electrophoresis and western blotting with antibodies against S antigen and from *E. coli* fractions the antigenic determinant forming neutralizing antibodies of S

antigen was verified to exist in the outer membrane (See Fig. 9A; the result of western blot in *E. coli* membrane fraction). In Fig. 9, lane 1 is JM 109 strain not transformed; lane 2, whole cell of the transformant pGNBCA-HB168/JM 109; lane 3, soluble fraction of the transformant pGNBCA-HB168/JM109; lane 4, the outer membrane fraction of the transformant pGNBCA-HB168/JM109; and lane 5, the outer membrane fraction of the transformant pGNBCA-HB168/JM109.

It is confirmed that the antigenic determinant forming neutralizing antibody of S antigen was expressed onto *E. coli* cell surface on account of the C-terminus of poly- $\gamma$ -glutamate synthetase protein, through the fluorescence activating cell sorting (FACS) flow cytometry. For an immunofluorescence staining, *E. coli* induced was harvested to adjust the cell concentration to the same level, washed with PBS buffer (pH 7.4) several times, suspended in 1 ml buffer containing 1% bovine serum albumin, and reacted with polyclonal primary antibody against S antigen derived from sheep after diluted to 1,000 X for 4 ~ 12 hours. The cells completed to react were washed several times in buffer solution, suspended in 1 ml buffer containing 1% bovine serum albumin and then reacted for 3 ~ 4 hours after diluting the biotin-associated secondary antibody. Again, the reacted cell was washed several times with buffer solution, suspended in 0.1 ml buffer containing 1% bovine serum albumin and then reacted with biotin-specific streptoavidin-R-phycoerythrin, coloring reagent after diluted to 1,000-fold.

The reacted *E. coli* was washed several times and examined through the fluorescence-activating cell sorting flow cytometry. As a result, the

antigenic determinant protein forming neutralizing antibody of S antigen was identified to be expressed onto a cell surface, compared with *E. coli* not transformed (See Fig. 9B). In Fig. 9B, white color is derived from JM 109 not transformed and black color, from the transformant pGNBCA-HB168/JM109.

5 As illustrated in Fig. 9, the antigenic determinant forming neutralizing antibody of S antigen was not expressed in *E. coli* not transformed, but in *E. coli* transformant transformed with the surface expression vector, the antigenic determinant forming neutralizing antibody of S antigen was clearly verified.

10

**<Indirect Example 2> Construction of the recombinant vector pGNCA-HB168 and surface expression of the antigenic determinant forming neutralizing antibody of hepatitis B virus S antigen**

15 Cell outer membrane gene, pgs C and pgs A among pgs BCA participating in poly- $\gamma$ -glutamate synthesis derived from *Bacillus sp.* strain was utilized to construct the recombinant vector for the surface expression by using Gram negative bacterium as a host cell.

20 In order to obtain the gene encoding the N-termini and the C-termini of pgs C and pgs A from cell outer membrane protein participating in poly- $\gamma$ -glutamate synthetase, whole chromosome was used as a template and oligonucleotides containing the nucleotide sequences of SEQ ID NO. 10 and SEQ ID NO. 11 as a primer. Then the polymerase chain reaction was performed.

The primer corresponding to the N-terminus was made to contain the recognition site of restriction enzyme Nde I present in the expression vector pHCE 19T(II). At this moment, the amplified region has approximately 1.6 kb size from the N-terminus of pgs C to the C-terminus of pgs A which is a cell  
5 outer membrane gene participating in poly- $\gamma$ -glutamate synthesis.

The gene amplified by the polymerase chain reaction was digested with the restriction enzyme Nde I and Bam HI and inserted to the constitutively high expression vector pHCE19T(II) already digested with Nde I and Bam HI. As a result, the new vector having about 5.3 kb size was  
10 prepared and named to pGNCA in which the cell outer membrane protein gene participating in the poly- $\gamma$ -glutamate synthesis do not have the translation termination codon and new recognition sites of restriction enzymes are added.

The recombinant vector pGNCA-HB168 expressing the antigenic  
15 determinant forming neutralizing antibody of hepatitis B virus S antigen onto the cell surface was constructed by using the cell outer membrane gene, pgs C and pgs A among pgs BCA participating in poly- $\gamma$ -glutamate synthesis and Gram negative bacterium as a host cell as described in Example 2. The recombinant vector pGNCA-HB168 prepared above was illustrated in Fig. 10.

20 The recombinant vector pGNCA-HB168 was utilized to produce the antigenic determinant forming neutralizing antibody of hepatitis B virus S antigen in *E. coli* and examined the surface expression.

For this purpose, the recombinant vector was transformed to *E. coli* and induced to be expressed through the same process described in

Example 3, and then, the antigenic determinant forming neutralizing antibody of hepatitis B virus S antigen fused with the cell outer membrane protein pgs CA was identified to be expressed by performing SDS polyacrylamide gel electrophoresis and western blotting with antibodies against S antigen.

5           In Fig. 11, lane 1 is host cell JM 109 not transformed; lane 2 the transformed pGNCA-HB168/JM109. As illustrated in Fig. 11, approximately 48 KDa band of fused protein from the plasmid pGNCA-HB168 was verified.

## 10       INDUSTRIAL APPLICABILITY

          The present inventors have developed a method for efficiently expressing capsid L1 protein of human papilloma virus onto a microbial surface by using poly- $\gamma$ -glutamate synthetase gene, pgs BCA derived from  
15       *Bacillus sp.* strain, especially onto a microbial surface of *Lactobacillus* orally administered and of *Salmonella* as a vaccine strain. Besides, the recombinant strain can be applied for a therapeutic and a prophylactic vaccine and the like. Since in case of HPV, the antibodies is not inductively produced in a large scale, the recombinant strain of the present invention  
20       expressing HPV antigen can be an economical vaccine such as an oral vaccine or directly applicable to vaginal legion, after proliferated in a large scale economically and chiefly.

          Those skilled in the art will appreciate that the conceptions and

specific embodiments disclosed in the foregoing description may be readily utilized as a basis for modifying or designing other embodiments for carrying out the same purposes of the present invention.

Those skilled in the art will also appreciate that such equivalent  
5 embodiments do not depart from the spirit and scope of the invention as set forth in the appended claims.


BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT  
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURES

INTERNATIONAL FORM

**RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT**

Issued pursuant to Rule 7.1

TO : **SUNG, Moon-Hye**  
DongA Venture Building 908,  
#338-8, Bongmyeong-dong, Yuseong-gu, Daejeon 305-709,  
Republic of Korea

<b>I. IDENTIFICATION OF THE MICROORGANISM</b>	
Identification reference given by the DEPOSITOR:  <i>Escherichia coli</i> DH5 $\alpha$ /pHCE2LB:pgsA-HPVL1	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:  KCTC 10949BP
<b>II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION</b>	
The microorganism identified under I above was accompanied by: <input checked="" type="checkbox"/> a scientific description <input type="checkbox"/> a proposed taxonomic designation (Mark with a cross where applicable)	
<b>III. RECEIPT AND ACCEPTANCE</b>	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on October 04 2002.	
<b>IV. RECEIPT OF REQUEST FOR CONVERSION</b>	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
<b>V. INTERNATIONAL DEPOSITARY AUTHORITY</b>	
Name: Korean Collection for Type Cultures  Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yuseong-gu, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):   PARK Yong-Ha, Director Date: October 09 2002



INTERNATIONAL TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT  
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

## INTERNATIONAL FORM

## RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : ROQ, Hanyoung  
Korea Research Institute of Bioscience and Biotechnology,  
#52, Oun-dong, Yusong-gu, Taejeon 305-383,  
Republic of Korea

## I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the  
DEPOSITOR:

*Escherichia coli*  
JM83/pHCE2LB-pgsBCA-HPVR7

Accession number given by the  
INTERNATIONAL DEPOSITARY  
AUTHORITY:

KCTC 10520BP

## II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☒ a scientific description  
☐ a proposed taxonomic designation  
(Mark with a cross where applicable)

## III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above,  
which was received by it on **October 07 2003**.

## IV. RECEIPT OF REQUEST FOR CONVERSION

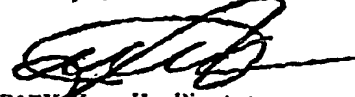
The microorganism identified under I above was received by this International Depositary  
Authority on \_\_\_\_\_ and a request to convert the original deposit to a deposit  
under the Budapest Treaty was received by it on \_\_\_\_\_

## V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures

Address: Korea Research Institute of  
Bioscience and Biotechnology  
(KRIH)  
#52, Oun-dong, Yusong-gu,  
Taejeon 305-383,  
Republic of Korea

Signature(s) of person(s) having the power-  
to represent the International Depositary  
Authority of authorized official(s):

  
PARK Yong Ha, Director  
Date: October 13 2003

**What is claimed is:**

1. A vector for preparing a vaccine which contains one or more than two gene among pgs B, pgs C and pgs A encoding poly- $\gamma$ -glutamate synthetase complex and an antigen protein gene of human papilloma virus:  
5
2. The vector for preparing a vaccine according to claim 1, in which said antigen protein gene is one or more than two genes selected from a group comprising capsid HPV L1 and HPV L2 of human papilloma virus.  
10
3. The vector for preparing a vaccine according to claim 1, in which said antigen protein gene is one or more than two genes selected from a group comprising HPV E6 and HPV E7 antigen protein associated with a tumor induction.  
15
4. The vector for preparing a vaccine according to claim 1, in which pgs A gene encoding said poly- $\gamma$ -glutamate synthetase complex is contained.
5. A Gram negative microbe which is transformed with the vector for  
20 preparing a vaccine of claim 1.
6. The microbe according to claim 5, in which said microbe is selected from a group comprising *Escherichia coli*, *Salmonella typhi*, *Salmonella typhimurium*, *Mycobacterium bovis*, and *Shigella*.

7. A Gram positive microbe which is transformed with the vector for preparing a vaccine of claim 1.

8. The microbe according to claim 7, in which said microbe is selected from a group comprising *Bacillus*, *Lactobacillus*, *Lactococcus*, *Staphylococcus*, *Lysteria*, *Monocytogenesis*, and *Streptococcus*.

9. A vaccine for treating or preventing mucosal tumor which contains as an effective component, the microbes of claim 5 and claim 7 expressing an antigen protein onto a cell surface, crude antigen proteins extract from said microbes or antigen proteins purified from said microbes.

10. The vaccine for treating or preventing mucosal tumor according to claim 9, which can be administered orally or be edible.

11. The vaccine for treating or preventing mucus tumor according to claim 9, which can be injected subcutaneously or peritoneally.

12. The vaccine for treating or preventing mucosal tumor according to claim 9, which can be sprayed to the nasal cavity.

13. The vector for preparing a vaccine according to claim 1, which has the genetic map as illustrated in Fig. 1 and is named as pHCE2LB:pgsA-HPV L1

14. The vector for preparing a vaccine according to claim 1, which has the genetic map as illustrated in Fig. 5 and is named as pHCE2LB:pgsBCA-HPV E7.

5 15. A microbe which is transformed with the vector for preparing a vaccine of claim 13 or claim 14.

16. The microbe according to claim 15, in which *Lactobacillus* or *Salmonella* is used as a host cell.

10

17. The *Escherichia coli* transformant which is transformed with the vector of claim 13 (accession number: KCTC 10349 BP)

15

18. The *Escherichia coli* transformant which is transformed with the vector of claim 13 (accession number: KCTC 10520 BP)

20

19. A vaccine for treating or preventing mucosal tumor which includes as an effective component, the microbes of claim 16 expressing an antigen protein onto a cell surface, crude antigen proteins extract from said microbes or antigen proteins purified from said microbes.

20. The vaccine for treating or preventing mucosal tumor according to claim 19, which can be administered orally or be edible.

21. The vaccine for treating or preventing mucosal tumor according to claim 19, which can be injected subcutaneously or peritoneally.
22. The vaccine for treating or preventing mucosal tumor according to  
5 claim 19, which can be sprayed to the nasal cavity.
23. A washing solution for a genital organ, which includes as an effective component, the microbes of claim 16 expressing an antigen protein onto a cell surface, crude antigen protein extract from said microbes or antigen  
10 proteins purified from said microbes.

1/14

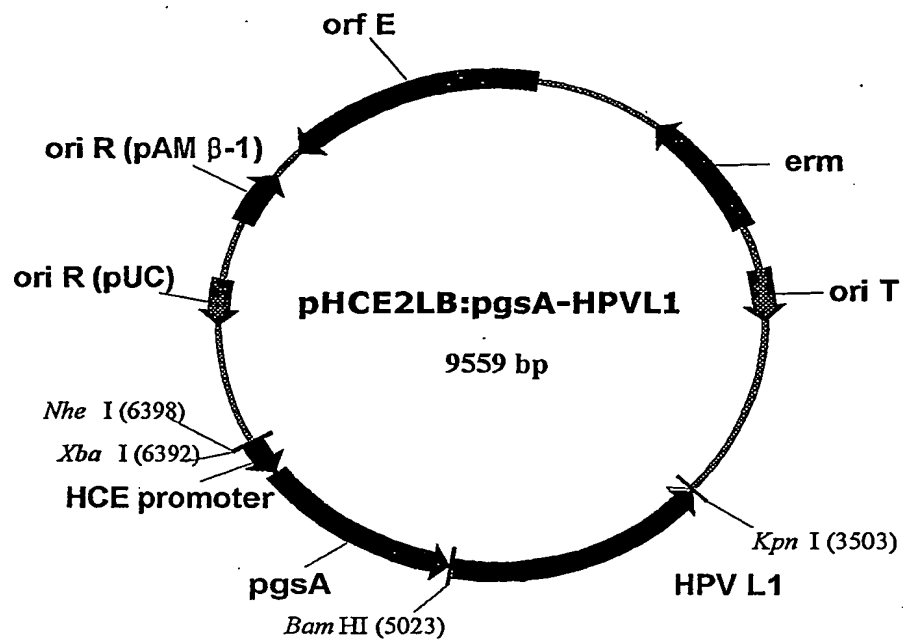


FIG 1

2/14

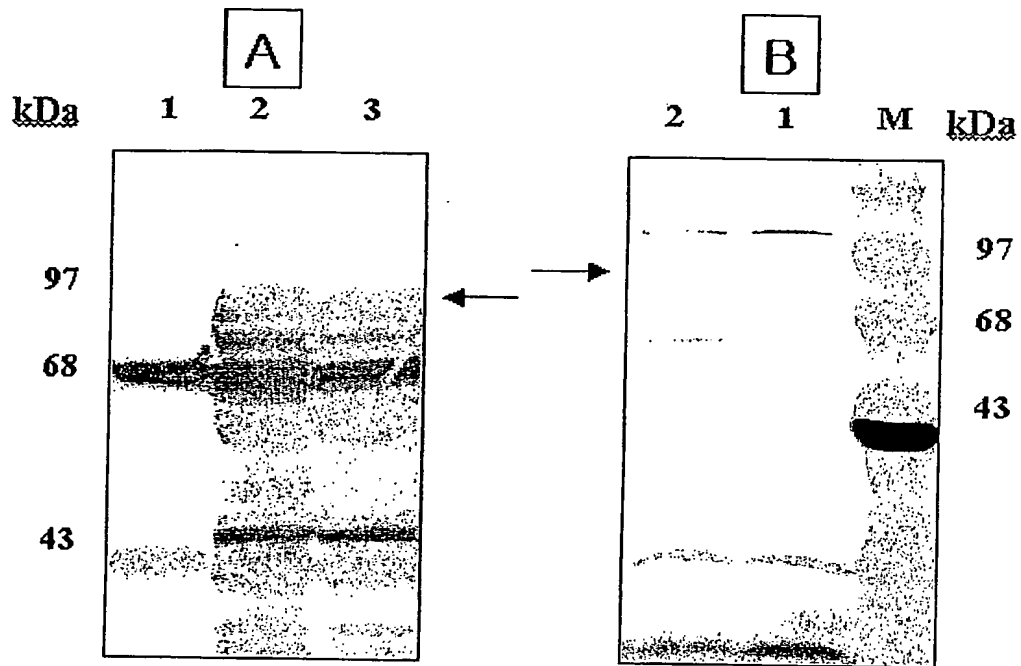


FIG 2

3/14

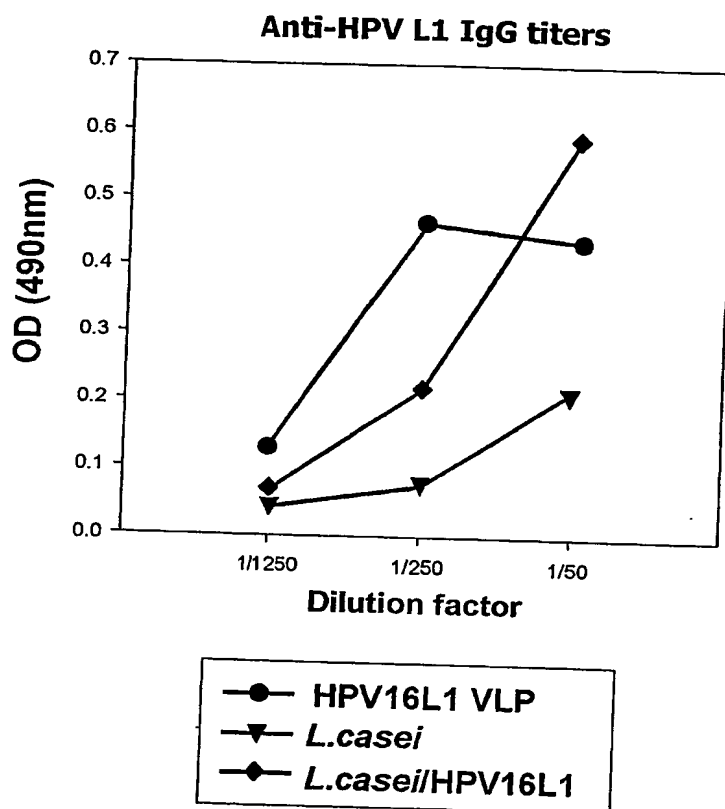
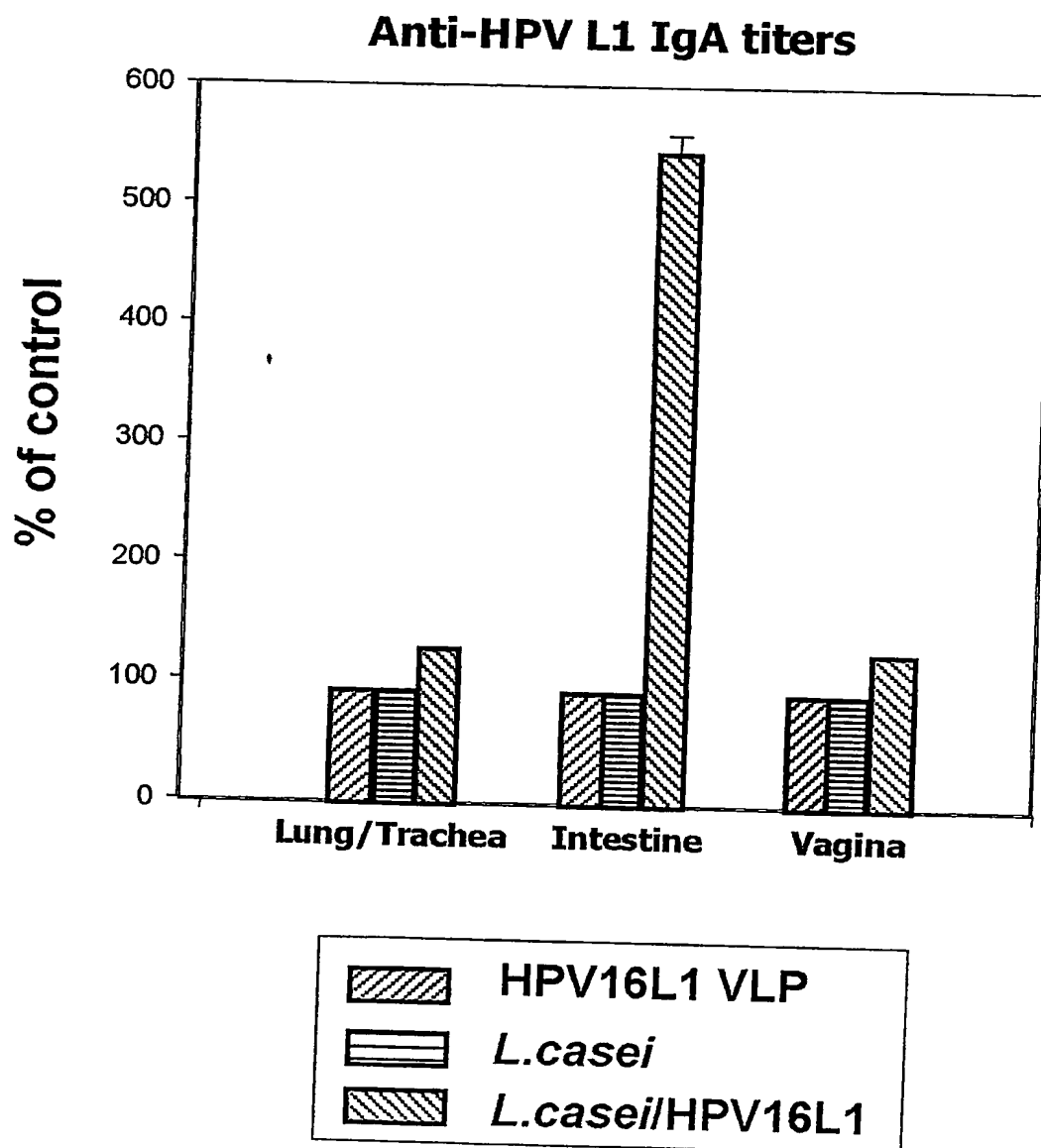


FIG 3A



4/14



5/14

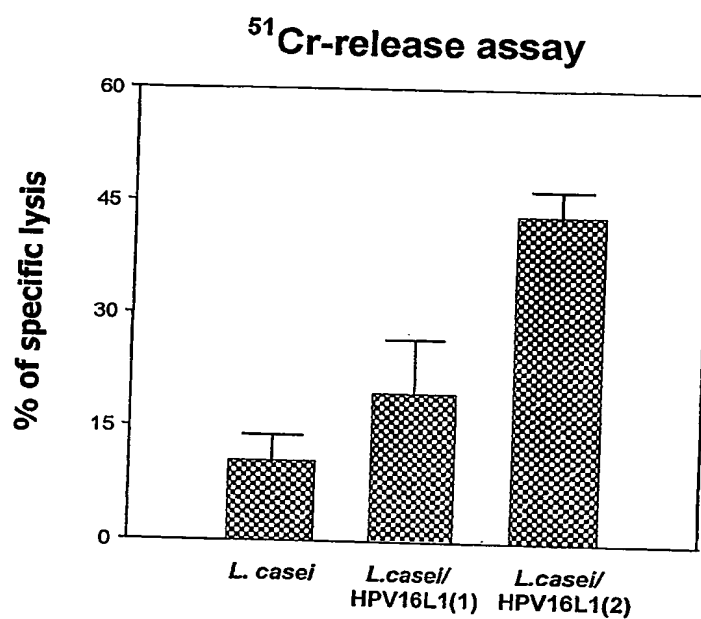


FIG 4

6/14

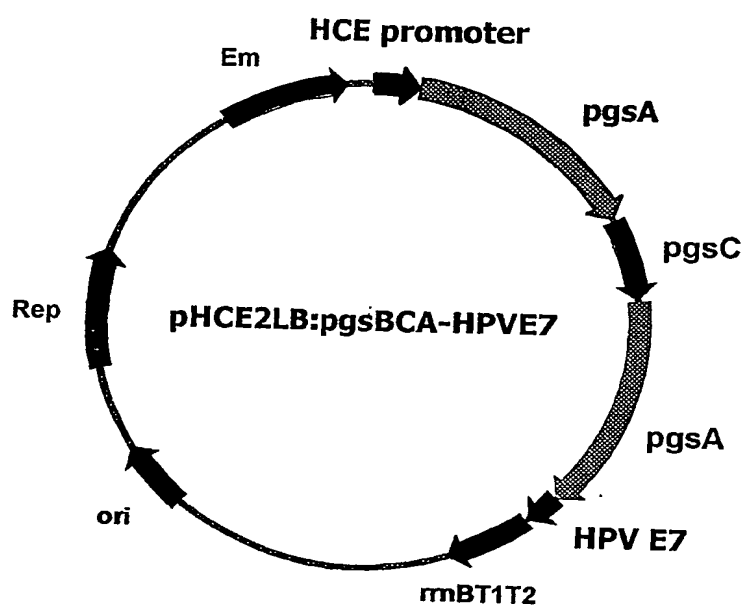


FIG 5

7/14

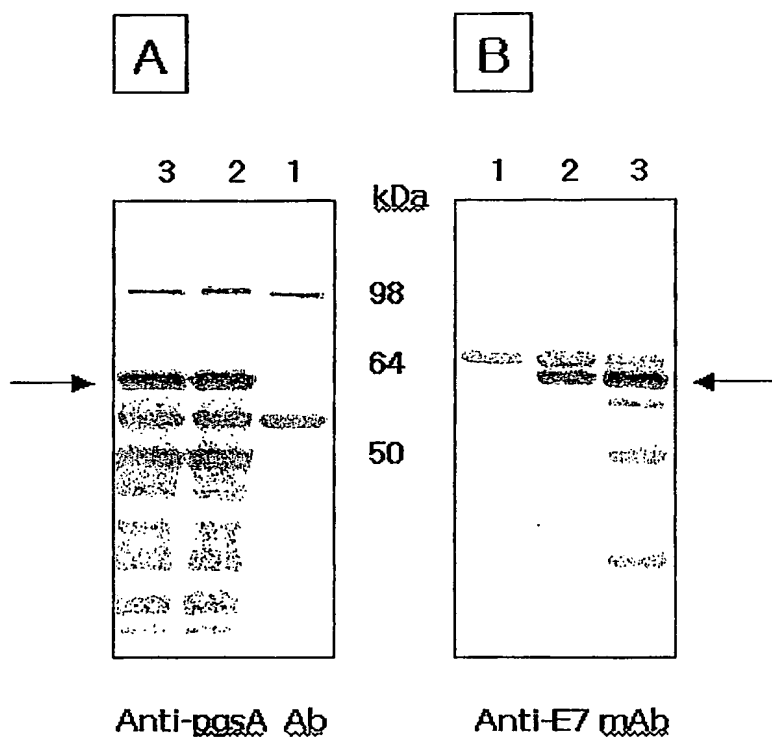
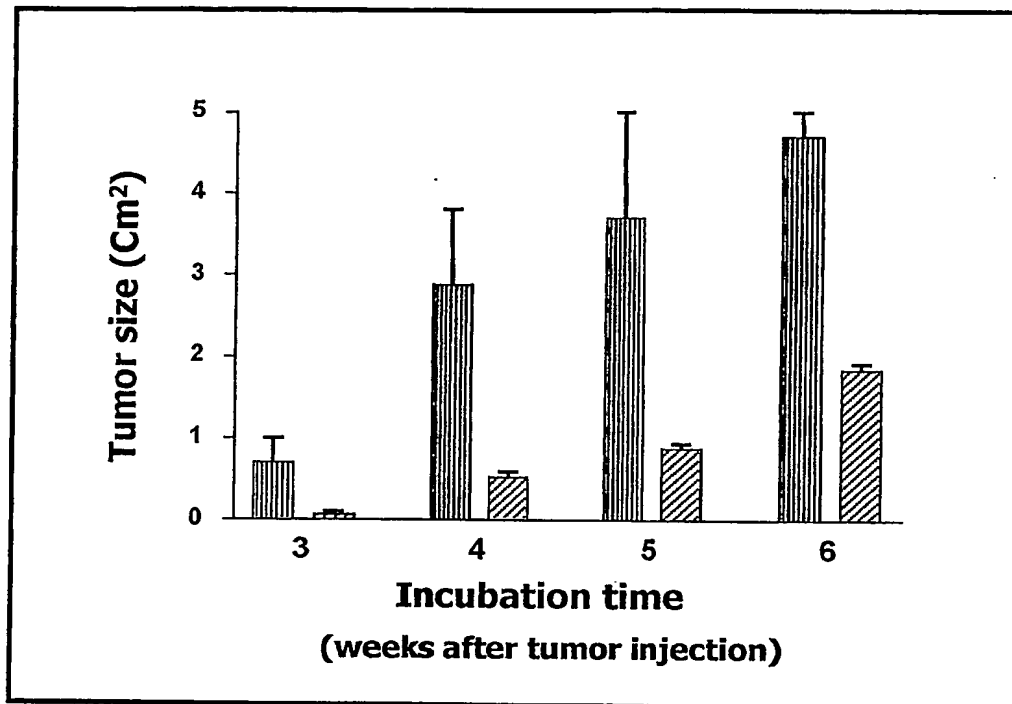


FIG 6

8/14



 *L.casei*  
 *L.casei*/HPV16E7

FIG 7

9/14

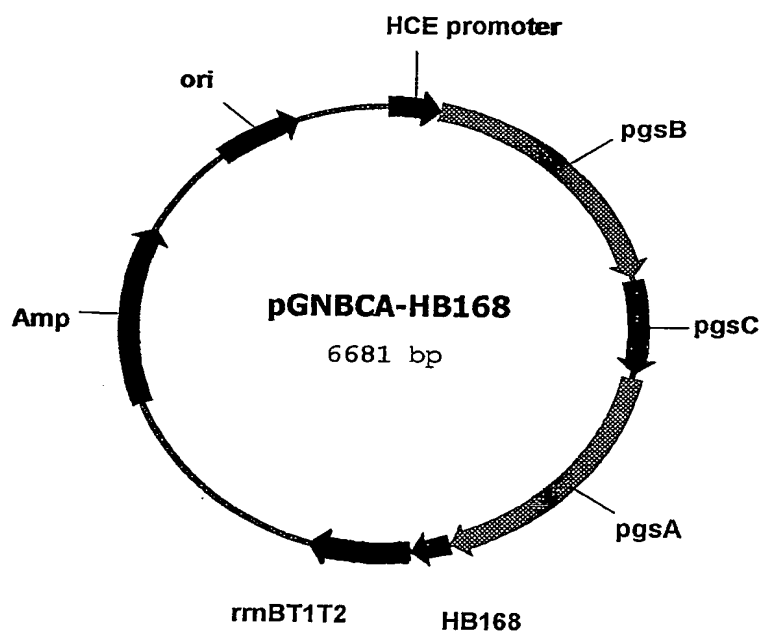


FIG 8

10/14

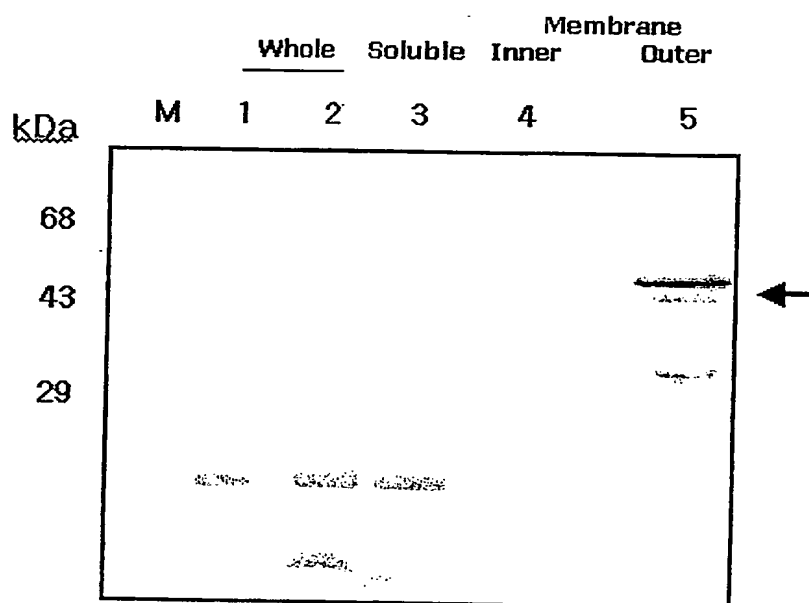


FIG 9A

11/14

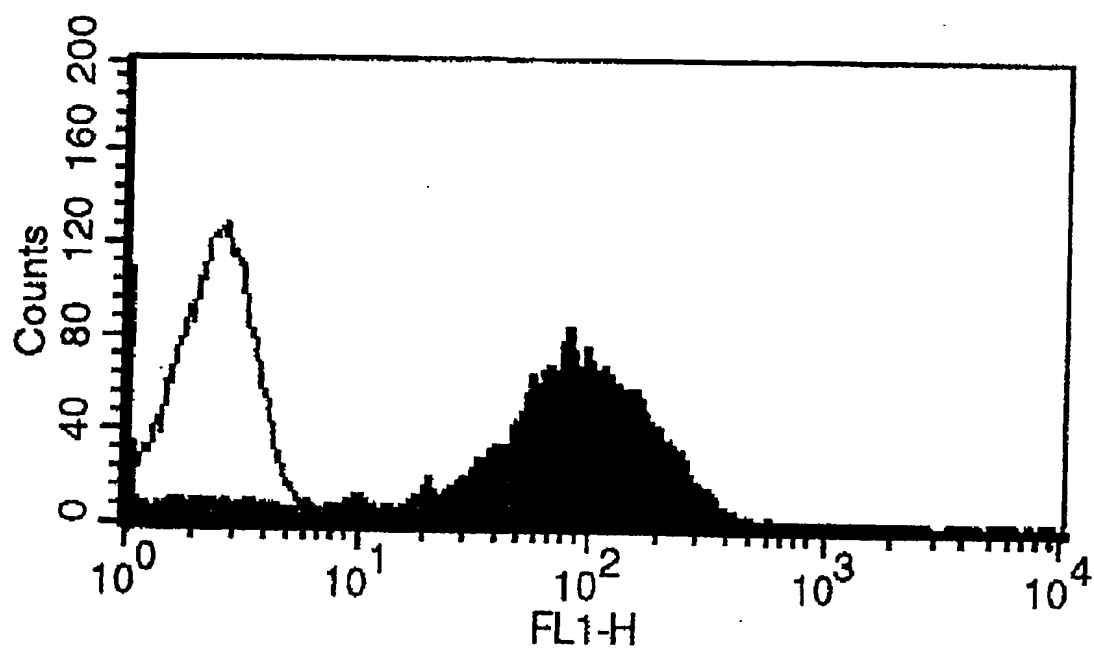
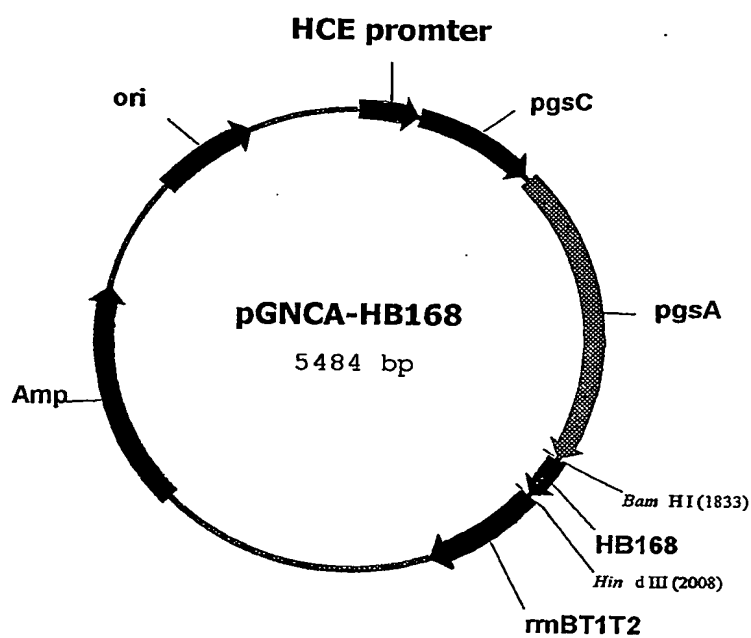


FIG 9B



12/14



**FIG 10**

13/14

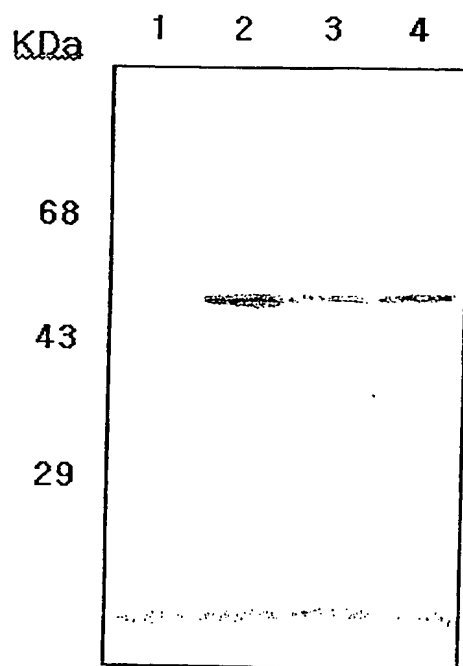


FIG 11A

14/14

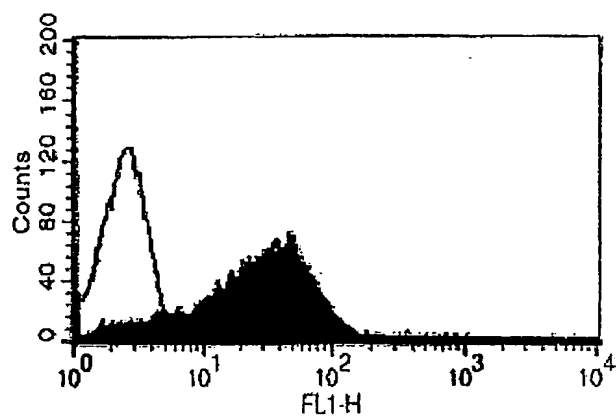


FIG 11B

## Sequence Listing

<110> BIOLEADERS CORPORATION  
KOREA RESEARCH INSTITUTE OF BIOSCIENCE AND BIOTECHNOLOGY  
SUNG, Moon-Hee  
POO, Ha-Ryoung  
LEE, Jong-Soo  
JUNG, Chang-Min  
HONG, Seung-Pyo  
KIM, Chul-Joong  
PARK, Sue-nie  
PYO, Hyun-mi

<120> VECTOR FOR ANTI-HPV VACCINE AND TRANSFORMED MICROORGANISM BY THE VECTOR

<130> E03-004

<150> KR10-2002-0063378  
<151> 2002-10-17

<160> 11

<170> KopatentIn 1.71

<210> 1  
<211> 1182  
<212> DNA  
<213> Bacillus subtilis

<400> 1  
atgggctggt tactcattat agcctgtgct gtcatactgg tcatcggaat attagaaaaa 60  
cgacgacatc agaaaaacat tgatgccctc cctgttcggg tgaatattaa cggcatccgc 120  
ggaaaatcga ctgtgacaag gctgacaacc ggaatattaa tagaagccgg ttacaagact 180  
gttggaaaaa caacaggaac agatgcaaga atgatttact gggacacacc ggaggaaaag 240  
ccgattaaac ggaaacctca ggggccgaat atcggagagc aaaaagaagt catgagagaa 300  
acagtagaaa gaggggctaa cgcgattgtc agtgaatgca tggctgttaa cccagattat 360

## Sequence Listing

---

```

caaatcatct ttcaggaaga acttctgcag gccaatatcg gcgtcattgt gaatgtttta      420
gaagaccata tggatgtcat ggggccgacg cttgatgaaa ttgcagaagc gtttaccgct      480
acaattcctt ataatggcca tcttgtcatt acagatagtg aatataccga gttctttaaa      540
caaaaagcaa aagaacgaaa cacaaaagtc atcattgtcg ataactcaaa aattacagat      600
gagtatttac gtaattttga atacatggta ttccctgata acgcttctct ggcgctgggt      660
gtggctcaag cactcggcat tgacgaagaa acagcattta agggaatgct gaatgcgccg      720
ccagatccgg gagcaatgag aattcttcgg ctgatcagtc cgagcgagcc tgggcacttt      780
gttaatgggt ttgccgcaaa cgacgcttct tctactttga atatatggaa acgtgtaaaa      840
gaaatcggtt acccgaccga tgatccgata atcatcatga actgcgcgcg agaccgtgtc      900
gatcggacac agcaattcgc aaatgacgta ttgccttata ttgaagcaag tgaactgac      960
ttaatcgggt aaacaacaga accgatcgta aaagcctatg aagaaggcaa aattcctgca     1020
gacaaactgc atgacctaga gtataagtca acagatgaaa ttatggaatt gttaaagaaa     1080
agaatgcaca accgtgtcat atatggcgtc ggcaatatte atggtgccgc agagccttta     1140
attgaaaaaa tccacgaata caaggtaaag cagctcgtaa gc                          1182

```

```

<210>      2
<211>     447
<212>      DNA
<213>      Bacillus subtilis

```

```

<400>      2
atgttcggat cagatttata catcgcactg attttaggtg tactactcag ttttaatttt      60
gcggaaaaaa cagggatcgt gccggcagga cttgtgttac cgggatattt aggacttgtg     120

```

## Sequence Listing

---

tttaatcagc cggctctttat ttactttgtt ttgctagtga gcttgctcac ttatgttacc	180
gtgaaatacg gtttatccaa atttatgatt ttgtacggac gcagaaaatt cgctgccatg	240
ctgataacag ggatcgtcct aaaaatcgcg ttgattttc tataccgat tgtaccattt	300
gaaatcgag aatttcgagg aatcggcac atcgtgccag gtttaattgc caataccatt	360
cagaaacaag gtttaaccat tacgttcgga agcacgtgc tattgagcgg agcgacctt	420
gctatcatgt ttgtttacta ctttaatt	447
<div style="display: flex; justify-content: space-between;"> <span>&lt;210&gt; 3</span> <span>&lt;211&gt; 1140</span> </div> <div style="display: flex; justify-content: space-between;"> <span>&lt;212&gt; DNA</span> <span>&lt;213&gt; Bacillus subtilis</span> </div>	
<div style="display: flex; justify-content: space-between;"> <span>&lt;400&gt; 3</span> </div>	
atgaaaaaag aactgagctt tcatgaaaag ctgctaagc tgacaaaaca gcaaaaaaag	60
aaaaccaata agcacgtatt tattgccatt ccgacgctt ttgtccttat gttcgcttcc	120
atgtgggctg gaaaagcggg aacgccgaag gtcaaacgt attctgacga cgtactctca	180
gcctcatttg taggcgatat tatgatggga cgtatgttg aaaaagtaac ggagcaaaaa	240
ggggcagaca gtatttttca atatgttgaa ccgatcttta gagcctcgga ttatgtagca	300
ggaaactttg aaaaccgggt aacctatcaa aagaattata aacaagcaga taaagagatt	360
catctgcaga cgaataagga atcagtgaaa gtcttgaagg atatgaattt cacggttctc	420
aacagcgcca acaaccacgc aatggattac ggcgttcagg gcatgaaaga tacgcttgga	480
gaatttgcca agcaaacct tgatatcgtt ggagcgggat acagcttaag tgatgcgaaa	540
aagaaaattt cgtaccagaa agtcaacggg gtaacgattg caacgcttgg ctttaccgat	600
gtgtccggga aaggtttcgc ggctaaaaag aatacgccgg gcgtgctgcc cgcagatcct	660

## Sequence Listing

gaaatcttca tccctatgat ttcagaagcg aaaaaacatg ctgacattgt tgttgtgcag 720  
tcacactggg gccaaagagta tgacaatgat ccaaacgacc gccagcgcca gcttgcaaga 780  
gccatgtctg atgcgggagc tgacatcatc gtcggccatc atccgcacgt cttagaaccg 840  
attgaagtat ataacggaac cgtcattttc tacagcctcg gcaactttgt ctttgaccaa 900  
ggctggacga gaacaagaga cagtgcactg gttcagtatc acctgaagaa aaatggaaca 960  
ggccgctttg aagtgcacc gatcgatc catgaagoga cacctgcacc tgtgaaaaaa 1020  
gacagcctta aacagaaaac cattattcgc gaactgacga aagactctaa ttctgcttgg 1080  
aaagtagaag acggaaaact gacgtttgat attgatcata gtgacaaact aaaatctaaa 1140  
1140

<210> 4  
<211> 26  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 4  
cgcgatcct ctctttggct gcctag 26

<210> 5  
<211> 31  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

## Sequence Listing

---

<400> 5

ggaaagcttt tattacagct tacgtttttt g

31

<210> 6

<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 6

cgcggatccc caggaggtat gcat

24

<210> 7

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 7

ggaaagcttt tatggtttct gagaacaga

29

<210> 8

<211> 29

<212> DNA

<213> Artificial Sequence

<220>

<223> primer



## Sequence Listing

<400> 8  
ctgggatccc aaggtatggt gcccgtttg

29

<210> 9  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 9  
tgaagcttat taggacgatg ggatgggaat

30

<210> 10  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

<400> 10  
gcacatatgt tcggatcaga ttatacatc

30

<210> 11  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> primer

## Sequence Listing

<400> 11

ctcggatcct ttagatttta gtttgtcact

30

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR2003/002163

## A. CLASSIFICATION OF SUBJECT MATTER

**IPC7 C12N 15/63, A61K 39/12, A61K 31/00**

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/63, 1/20, A61K 39/12, 31/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubMed, Delphion, CA, "pgsB", "pgsC", "pgsA", "pgsBCA", "HPVL", "HPVE", "vaccine"

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 1999/001557 A1 (UNIVERSITY OF COLORADO et al.) 14 January 1999. See the whole document.	9-12, 19-23
X	WO 2000/075336 A1 (BIOVECTOR THERAPEUTICS et al.) 12 December 2000. See the whole document.	9-12, 19-23
X	Lehtinen, M. and Paavonen, J., 'Efficacy of preventive human papillomavirus vaccination.', Int. J. STD. AIDS., 12(12):771-776, December, 2001. See the whole document.	9-12, 19-23
A	Ashiuchi, M. et al., 'A poly-r-glutamate synthetic system of Bacillus subtilis IFO 3336: Gene cloning and biochemical analysis of poly-r-glutamate produced by Escherichia coli clone cells.' Biochem. Biophys. Res. Commun., 263(1):6-12, 1999. See the whole document.	1-8, 13-18
A	JP 2001017182 A2 (Nagase Co., Ltd.), 23 January 2001. See the whole document.	1-8, 13-18
T	Bubenik, J., 'Therapeutic vaccines against HPV16-associated tumors. Minireview', Neoplasma, 49(5):285-289, 2002. See the whole document.	9-12, 19-23

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

17 JANUARY 2004 (17.01.2004)

Date of mailing of the international search report

19 JANUARY 2004 (19.01.2004)

Name and mailing address of the ISA/KR



Korean Intellectual Property Office  
920 Dunsan-dong, Seo-gu, Daejeon 302-701,  
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LEE, CHUNG HO

Telephone No. 82-42-481-8160



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2003/002163

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 1999/001577 A1	14.01.1999.	US 9813799	14.01.1999.
		AU 8284298	25.01.1999.
		EP 1000127	17.05.2000.
		JP 2002510976	09.04.2002.
		AU 755679	19.12.2002.
WO 2000/075336 A1	14.12.2000.	FR 2794370	08.12.2000.
		WO 0075181	14.12.2000.
		AU 5229400	28.12.2000.
		AU 5411000	28.12.2000.
		EP 1181314	27.02.2002.
JP 2001017182 A2	23.01.2001.	None	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2003/002163

## Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of :

a. type of material

- ☒ a sequence listing  
☐ table(s) related to the sequence listing

b. format of material

- ☒ in written format  
☒ in computer readable form

c. time of filing/furnishing

- ☒ contained in the international application as filed  
☒ filed together with the international application in computer readable form  
☐ furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments: